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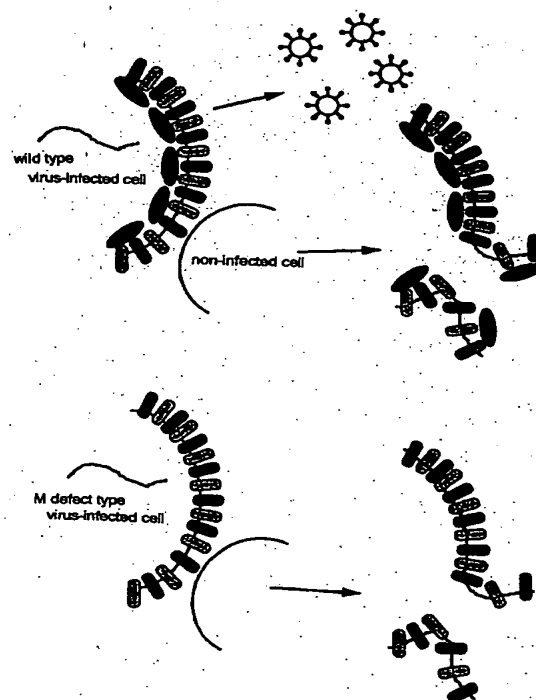
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**(54) RNA VIRUS VECTOR HAVING CONTACT INFILTRATION CAPABILITY**

(57) The present invention provides an RNA virus vector useful for gene therapy. The RNA virus vector is capable of cell infection, autonomous RNA replication, and infiltration through contact, but incapable of dissemination. The use of the RNA virus vector of the present invention enables more efficient gene transfer and cell transplantation in gene therapy compared with the conventional techniques.

Fig. 9



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**Description****Technical Field**

**[0001]** The present invention relates to a virus vector that can be used for gene therapy, and more specifically, to an inactivated negative strand RNA ((-)RNA) viral vector.

**Background Art**

**[0002]** In gene therapy for humans and animals, effectiveness and safety are very important. Particularly, in therapy using a "virus vector" which is obtained by viral gene recombination, treatment must be carried out with extreme care, when it is hard to discriminate the possibilities of nonspecific chromosomal integration, and leakage of recombinant or pathogenic viruses to nature, uncontrolled expression of an introduced gene even if the treatment is effective.

**[0003]** Virus vectors are mainly used for introducing a gene of interest into target cells by utilizing viral infectivity. Recombinant virus vectors, which have been genetically engineered to carry a gene insert(s), contain viral envelope proteins on the surface. Since the envelope proteins retain viral infectivity, it is possible to introduce its endogeneous recombinant gene inserts into cells. These vectors can be utilized for not only gene therapy but also production of cells expressing a gene of interest and creation of transgenic animals.

**[0004]** Virus vectors are classified into three: retrovirus vectors, DNA virus vectors, and RNA virus vectors. Among them, RNA virus vectors which are not integrated into chromosomes, are advantageous in terms of safety. According to this technical background, virus vectors derived from non-segmented (-) RNA virus such as Sendai virus have already been provided (D. Yu et al., Genes to Cells, 2:457-466, 1997; M. Hasan et al., J. Gen. Virol. 78:2813-2820, 1997). The present inventors have developed a (-)RNA virus vector that has infectivity and autonomous RNA replication ability without disseminative capability (WO 97/16538).

**Disclosure of the Invention**

**[0005]** An objective of the present invention is to provide an RNA virus vector that can be used for gene therapy.

**[0006]** Using nucleic acid of Sendai virus, a representative of (-)RNA viruses, which is supposed to be the most useful vector for industrial applications in terms of safety and convenience, the present inventors succeeded in obtaining various defective mutants, which was experimentally reconstituted using known methods. As a result, the inventors found that the complexes reconstituted by the mutants carrying a defect in M gene were capable of forming plaques, which were smaller than those formed by wild type viruses, but incapable of propagating in embryonated chicken eggs. Thus, the inventors confirmed that the mutants are capable of cell infection, autonomous RNA replication, and contact infiltration, but incapable of dissemination. Furthermore, the plaques formed by a defective mutant carrying defect in the M gene were stained with anti-Sendai virus antibody, but not with anti-M monoclonal antibody. From this result the inventors confirmed that plaques formed by the M mutants are lacking complete M protein, thereby accomplishing the present invention.

**[0007]** Thus, the present invention provides RNA comprising genes involved in contact infiltration and autonomous RNA replication, but no or inactivated genes involved in dissemination.

**[0008]** The present invention also provides a cell comprising the above-described RNA, which is capable of allowing the RNA to replicate therein and transmitting said RNA to another cell through contact infiltration.

**[0009]** Furthermore, the present invention provides a complex capable of cell infection, contact infiltration, and autonomous RNA replication but incapable of dissemination, a method for producing the complex, and a kit and host for producing the complex.

**[0010]** Moreover, the present invention provides a DNA comprising a template DNA for transcribing the above-described RNA *in vitro* or in a cell.

**[0011]** Herein, "cell infectivity" of a virus vector means "an ability of a viral vector capable of cell adhesion and membrane fusion to introduce its endogeneous nucleic acids into cells." "Disseminative capability" means "an ability of a nucleic acid introduced into a cell artificially or by infection to form infective particles or comparable complexes following replication of said nucleic acid in the cell, and then be transmitted into another cell." "Contact infiltration capability" means "an ability of a cell carrying a viral vector gene to transfer the gene to another cell through contact."

**[0012]** A viral vector capable of contact infiltration but incapable of dissemination can overcome a critical demerit of existing virus vectors, which are incapable of dissemination and contact infiltration. In other words, when cells are infected with a virus vector to express a gene insert in the virus vector, the existing virus vectors are only able to express a protein in cells directly infected. This is not a problem when cell culture systems are used, in which the cells grow in a layer and can be directly infected with viral vectors. However, when *in vivo* cells that exist three-dimensionally in multi-layers are infected with viral vectors, the region and the number of cells directly infected by the existing vectors

are limited. For example, it is extremely difficult to directly infect all tumor-forming cells at a tumor site with the viral vectors.

[0013] By contrast, even if virus vectors capable of contact infiltration of the present invention is used to infect tumor cells at a tumor site and only a part of the cells are directly infected, noninfected cells adjacent to the infected cells can be infected with the vectors through contact infiltration, resulting in infecting all the tumor cells. Since the vector is not disseminative, there is no possibility that the vector infects other cells in the body through blood stream.

[0014] Thus, the virus vector of the present invention has such novel features that it infects all the cells in a restricted region or a particular organ and does not infect the other part of the body.

[0015] Furthermore, cells carrying the virus vector of the present invention capable of cell infection, autonomous RNA replication, and contact infiltration, but incapable of disseminative, can be utilized for cell transplantation. Since a cell carrying a virus vector capable of autonomous replication and contact infiltration can transfer the vector to another cell, it is possible to transplant the cell carrying the vector at the minimal dose to a diseased site, thereby treating there.

[0016] It has been reported that the formation of infective particles of Measles virus having a defect in the M gene is inhibited, and cell fusion is promoted (T. Cathomen et al., EMBO J., 17:3899-3908, 1998). However, the possibility to utilize the virus as a vector has not been suggested.

[0017] A source of a (-)RNA virus of the present invention includes Sendai virus, Newcastle disease virus, Mumps virus, Measles virus, Respiratory syncytial virus, rinderpest virus, and distemper virus, of the Paramyxoviridae; influenza virus of the Orthomyxoviridae, and Vesicular S, and Rabies virus of the rhabdoviridae. Incomplete viruses such as DI particle (J. Virol. 68:8413-8417, 1994) and synthetic oligonucleotides can also be used.

[0018] A recombinant (-) RNA virus derived from any viruses described above can also be used as a vector source. For example, the recombinant (-)RNA viruses may be those with the gene for antigenicity inactivated or those with some genes altered to improve efficiency of RNA transcription or replication.

[0019] An RNA of the present invention can be obtained by transcribing an altered cDNA derived from any viruses or recombinant viruses described above in vitro or in cells. In the obtained RNA, at least one gene involved in disseminative capability of the original virus must be deleted or inactivated, but gene involved in autonomous replication and contact infiltration should not be deleted or inactivated. In addition, an RNA molecule with artificial sequences, which are prepared by transcribing *in vitro* or in cells a DNA obtained by inserting genes for autonomous replication into a cDNA containing the structure of both ends of a virus genome such as DI molecules can also be used.

[0020] In Sendai virus, "genes involved in autonomous replication" are either of NP, P/C, or L genes, and "genes involved in transmission" are either of M, F, or HN genes. When only the M gene is deleted or inactivated, "disseminative capability" will be lost, but "contact infiltration ability" will remain because virion or virus-like particles capable of releasing the complex containing genomic RNA extracellularly are not formed. Therefore, for example, an RNA of Sendai virus Z strain deficient in only the M gene and a ribonucleoprotein (RNP) comprising said RNA are suitably used in the present invention. The genes are not necessary to be the same as the original viral sequences. They can be mutated or substituted by the corresponding gene of other viruses as long as the resulting gene has transcription and replication activities comparable to or higher than those of the wild type.

[0021] An RNA of the present invention may have a foreign gene inserted to an appropriate site. To express a desired protein, a foreign gene encoding the protein is inserted. In the case of Sendai virus RNA (GenBank accession No. M30202), a sequence with a multiple of six bases between the R1 and R2 sequences (J. Virol., Vol. 67, No. 8, 1993, 4822-4830). The consensus sequences of R1 and R2 are 5'-AGGGWBAAWGD-3' and 5'-DTAAGAAAAA-3', respectively. The expression level of the inserted foreign gene can be regulated by the inserted position or the RNA nucleotide sequences flanking the inserted gene. For example, it is known about Sendai virus RNA that the expression level of the inserted gene is higher as the insert position is nearer to the NP gene.

[0022] A complex of the present invention comprises a virus structure without the above-described RNA and nucleic acid. "A virus structure without nucleic acid" is, for example, a virus from which only RNA is removed and complements infectivity and autonomous replication ability of the RNA of the present invention, not complement ability to form particles that release the complex extracellularly and move freely, in other words, not complement the disseminative capability. In Sendai virus, a complex of the RNA in which only the M gene is deleted and the virus structure from which the RNA and M protein are removed (RNP), has infectivity and autonomous replication ability, but not disseminative capability. The complex can contain any other components as long as they do not exhibit disseminative capability. For example, molecules that binds to a specific cell, such as adhesion molecules, ligands, or receptors, can be included on the envelope surface.

[0023] Moreover, the present invention relates to a kit comprising  
a) the above-described RNA of the present invention, a cRNA of said RNA, or a unit that is capable of biosynthesizing said RNA or said cRNA, and b) a group of enzymes required for replication of said RNA or said cRNA, or a unit that is capable of biosynthesizing said enzymes. The complex of the present invention, which is capable of cell infection, autonomous RNA replication, and contact infiltration, but incapable of dissemination, can be produced by introducing units of a) and b) into an appropriate host. Producing the complex of the present invention means reconstituting an

RNP having autonomous replication ability. When RNA derived from Sendai virus is used for a), b) is preferably all of NP, P/C, and L proteins derived from Sendai virus or a unit capable of biosynthesizing said proteins.

[0024] A host is not limited as long as it allows virus RNA used to be expressed therein. When RNA derived from Sendai virus is used, any cells susceptible to the infection with Sendai virus can be used, including cultured mammalian cells, cultured avian cells, and embryonated chicken eggs. Examples of cultured cells are LLCMK2, MDCK, MDBK, CV-1, Hela, HepG2, P19, F9, CHO, PC12, 293 cell, BAF3, Jerkat, human PBMC, MT-4, Molt-4, NIH3T3, L929, chicken embryo fibroblast, etc.

[0025] The present inventors have shown that Sendai virus cDNA reconstitutes particles more efficiently when cDNA to be introduced into cells is in a linear form than in a circular form, and that transcription of a (+)RNA in cells more successfully forms particles than that of a (-)RNA (A. Rato et al., Genes to Cells, 1: 569-579, 1996). Although these conditions may not always be applicable to the reconstitution of all other (-)RNA viruses, it is possible to determine the appropriate conditions for the reconstitution of other (-)RNA viruses according to the description of the present specification and based on conventional technology.

[0026] The produced complex can be recovered by the standard methods from the host, for example, cultured cells or embryonated chicken eggs.

[0027] A foreign gene encoded by the RNA constituting a complex can be expressed by infecting cells with the complex. Namely, the foreign gene can be expressed *in situ* by inoculating the complex or a cell carrying it into a host such as embryonated chicken eggs or appropriate tissues of an animal body. Alternatively, cells recovered from a living body are transformed so that the complex can autonomously replicate, and the transformed cells are returned to the living body to allow the cells to express the foreign gene.

[0028] As an embodiment of the present invention, Figure 9 shows the processes of infecting target cells with a Sendai virus complex in which the M gene is deleted or inactivated and infiltrating the RNA of the present invention into surrounding non-infected cells through contact infiltration.

#### Brief Description of the Drawings

[0029] Figure 1 schematically shows the structure of the plasmid pUC18/T7(+)-HVJRz.

[0030] Figure 2 schematically shows the region carrying the M gene in Sendai virus cDNA and procedure for subcloning or mutating said region. In the figure, E stands for transcription termination sequence; I, intervening sequence; and S, transcription initiation sequence. Boxes indicate the restriction sites used for the plasmid construction.

[0031] Figure 3 schematically shows procedures for introducing mutation to the M gene region of Sendai virus cDNA. Upper panel of A shows the structure of Sendai virus genome with each position of the NP, P (P/C), M, F, HN, and L genes. Lower panel shows the structure of the M gene, restriction sites, and positions of primers M1 and M2 used for the construction of the M defect type. B and C schematically show the construction of the M defect type and the M deletion type, respectively. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes. D shows comparison between the M defect and wild type M genes in terms of both nucleotide and amino acid sequences. Dots in the M defect sequences mean that bases or amino acids are identical to the corresponding wild type sequences. [Ter] indicates a termination codon. Numerals indicate the base/amino acid position in the M gene sequence.

[0032] Figure 4 schematically shows procedure for subcloning the M gene region of Sendai virus cDNA. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes.

[0033] Figure 5 schematically shows the construction of the M deletion type cDNA by mutating the subcloned Sendai virus M gene region and replacing the full-length M gene region of Sendai virus cDNA with the mutated one. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes.

[0034] Figure 6 schematically shows the construction of the M defect type cDNA by mutating the subcloned Sendai virus M gene region, and replacing the full-length M gene region of Sendai virus cDNA with the mutated one. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes.

[0035] Figure 7 shows plaques of Sendai virus cDNA of wild type (WT1 and WT2); M defect type (dM); and M deletion type (dMEA2-1 and dMEA2-2).

[0036] Figure 8 shows plaques of Sendai virus cDNA of wild type (WT); M defect type (dM); and M deletion type (dMEA), which were stained with anti-Sendai virus antibody.

[0037] Figure 9 illustrates the process of contact infiltration by Sendai virus genome of wild type genome (upper panel) and of the mutant with mutated or no M gene (lower panel). The wild type forms infective virus particles and causes contact infiltration, while the mutant is not able to form infective particles but introduces its genome into non-infected surrounding cells only by contact infiltration.

Best Mode for Carrying out the Invention

**[0038]** The present invention will be illustrated in detail below with reference to Examples, but is not to be construed as being limited thereto.

Example 1. Subcloning of the M gene of Sendai virus

**[0039]** All of the following ligation, blunting, and dephosphorylation were performed using the Takara Ligation Kit Ver. 2 (Takara Shuzo, Kyoto, Japan), the Takara Blunting Kit (Takara Shuzo), and CIAP (Takara Shuzo), respectively, according to the manufacture's protocols attached to the products. The nucleotide sequences were determined by Sanger's method (F. Sanger, Science, 214: 1205-1210, 1981).

**[0040]** Complementary DNA in which the M gene was deleted was constructed as follows, using wild type cDNA, pUC18/T7(+)-HVJRz (A. Kato et al., Genes to Cells, 1: 569-579, 1996) (Figure 1). The DNA sequences of the M gene and its vicinity are shown in Figure 2, and the construction scheme is shown in Figure 3.

**[0041]** First, the ClaI fragment containing the M gene was excised from pUC18/T7(+)-HVJRz and inserted into the ClaI site of pHSG396 (Takara Shuzo) to obtain pHSG-M-CC (Figure 4). Separately, pHSG396 was digested with ApaLI, blunted, and self-ligated to obtain pHSG396-dA that lacks the ApaLI site (Figure 4). The EcoRI-BamHI fragment of pHSG-M-CC carrying the M gene was inserted into dephosphorylated EcoRI-BamHI-digested pHSG396-dA to obtain pHSG-dA-M-BE (Figure 4).

Example 2. Construction of M deletion type vector

**[0042]** Forty nine-mer synthetic oligonucleotides, 5'-ggcgatatctatagattccctaagtctcatagtagatgtgcaccggca-3' (EA linker F) (SEQ ID NO: 1) and 5'-tgccggtgcacatctactatgagaacttaggaatctatagatcgcc-3' (EA linker R) (SEQ ID NO: 2), were annealed and inserted into pCR2.1 (Invitrogen) to generate pCR-EAL. The nucleotide sequence of the insert was verified to be as designed.

**[0043]** The pCR-EAL was digested with EcoRV and ApaLI, and the linker fragment was excised. Separately, the pHSG-dA-M-BE was digested with EcoRV and ApaLI and the above linker fragment was inserted therein to obtain pHSG-dA-dM-EA-EB.

**[0044]** The pHSG396 was digested with EcoRI, blunted, and self-ligated to obtain a plasmid lacking the EcoRI site, which was further digested with BamHI, blunted, and self-ligated to obtain pHSG-dE-dB, a plasmid lacking both EcoRI and BamHI sites.

**[0045]** The ClaI fragment carrying the M gene was inserted into the ClaI site of pHSG-dE-dB to generate pHSG-dE-dB-M-CC. The EcoRI-BamHI fragment excised from pHSG-dA-dM-EA-EB was inserted into the EcoRI-BamHI-digested pHSG-dE-dB-M-CC to obtain pHSG-dE-dB-dM-CC.

**[0046]** The ClaI fragment of pHSG-dE-dB-dM-CC was inserted into the ClaI site of the pUC18/T7(+)-HVJRz to obtain pHVJ-dMEA. This construction scheme is shown in Figure 5.

Example 3. Construction of M defect type vector

**[0047]** PCR was performed using pUC18/T7(+)-HVJRz as a template with oligonucleotide primers, M-1 (5'-ttaaaggcctaaaccgatctcagaattacg-3') (SEQ ID NO: 3), and M-2 (5'-tatcattccctgtctcagcctgcc-3') (SEQ ID NO: 4). The primers were designed to introduce a mutation at the BsgI site of the M gene so that a stop codon appears in frame of the M gene. The PCR products were subcloned into pCR2.1 to obtain pCR-M (Figure 6).

**[0048]** Following verification of the nucleotide sequence of pCR-M, the StuI-XcmI fragment of pCR-M was recovered from the gel and inserted into the StuI-XcmI-cut pHSG-M-CC. The resulting plasmid was named as pHSG-dM-CC. The ClaI fragment of pHSG-dM-CC was inserted into the ClaI site of pUC18/T7 (+) HVJRz to obtain pHVJ-dM. This construction scheme is shown in Figure 6.

Example 4. Reconstitution of virions from M deletion type and M defect type cDNAs

**[0049]** Reconstitution of virions from cDNA of wild type (WT), M deletion type (dMEA), and M defect type (dM) was performed as follows.

**[0050]** Cultured LLCMK2 cells (a simian kidney cell line) were detached from a dish by treatment with trypsin, and  $2 \times 10^6$  cells were plated in a plastic dish of 10 cm diameter and incubated in MEM medium (Gibco BRL) supplemented with 10% FBS (Gibco BRL) at 37°C for 24 hours in 5% CO<sub>2</sub>. The medium was then removed, and the cells were washed once with PBS. Five hundred  $\mu$ l of a solution of vTF7-3, a recombinant vaccinia virus vector capable of expressing T7 phage RNA polymerase (T.R. Fuerst et al., Proc. Natl. Acad. Sci. USA, 83:8122-8126, 1986), which was diluted with

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PBS to  $8 \times 10^6$  pfu/ml to obtain MOI (multiplicity of infection) of 2, was added dropwise to the dish. The cells were infected for 1 hour with agitating the dish every 15 minutes to spread the virus solution over the whole dish.

[0051] Separately, a medium containing a cDNA solution was prepared as follows. First, 500  $\mu$ l Opti-MEM (Gibco BRL) without FCS (fetal calf serum) was added into a polystyrene tube, and then any of pUC18/T7 (+) HVJRz, pHVJ-dMEA or pHVJ-dM, all plasmids originating from Sendai virus cDNA (20  $\mu$ g), pGEM-NP (5  $\mu$ g), pGEM-P (2.5  $\mu$ g), and pGEM-L (5  $\mu$ g) were added. Fifty  $\mu$ l SuperFect (QIAGEN) was further added, and the mixture was allowed to stand at room temperature for 20 minutes. Ten ml of Opti-MEM was then added. Subsequently, Rifampicin and Cytosine arabinoside (Ara C) were added at the final concentration of 100  $\mu$ g/ml and 40  $\mu$ g/ml, respectively.

[0052] After 1-hour infection, the virus solution was removed from the above dish containing LLCMK2 cells, the medium containing the above cDNA solution was added to the culture by decantation, and the cells were incubated at 37°C for 48 hours in 5% CO<sub>2</sub>. The cells were scraped using a cell scraper without removing the medium, and the cells and the medium were transferred together to a 15-ml centrifuge tube. After centrifugation at 1200 rpm for 5 minutes, the supernatant was removed, and the precipitated cell was resuspended in 200  $\mu$ l of PBS.

### Example 5. Inoculation of cell suspensions to embryonated chicken eggs and HA assay

[0053] To evaluate virus reconstitution, an assay using embryonated chicken eggs was performed. The cells suspended in 200  $\mu$ l of PBS obtained in Example 4 were adjusted to  $1 \times 10^7$  cells/ml and sequentially diluted with PBS to obtain cell suspensions of  $1 \times 10^6$  and  $1 \times 10^5$  cells/ml. One hundred  $\mu$ l of each suspension was inoculated to a 10-day-old embryonated chicken egg through the air chamber side using a 24G needle.

[0054] The chicken eggs were incubated at 35.5°C for 3 days with rotating. Then, the eggshell at the air chamber was cut out, and urine was collected using a 10-ml syringe with 18G needle.

[0055] The collected urine was tested by HA assay to evaluate virus propagation in the chicken eggs. HA assay was performed as follows.

[0056] Fifty  $\mu$ l of PBS was dispensed into each well of from the second to the 12th lines of a 96-well round bottom plate. One hundred  $\mu$ l of the urine sample was added to the first line. Fifty  $\mu$ l of the sample in the first line was mixed with PBS in the second line to make a 1:2 dilution. Fifty  $\mu$ l of the 1:2 dilution was mixed with PBS in the third line to make a 1:4 dilution. By repeating the procedure, a series of two-fold dilutions were made.

[0057] Fifty  $\mu$ l of 1% chicken preserved blood (Cosmo Bio) diluted with PBS was added to all the lines, and the plate was kept at 4°C for 1 hour. Erythrocyte aggregation was examined with the naked eye and the highest dilution that results in the aggregation was shown as HA activity (Table 1).

Table 1

Virus cDNA	HA activity
WT (wild type)	> 16
dM (defect)	< 2
dMEA2-1 (deletion)	< 2
dMEA2-2 (deletion)	< 2

[0058] Virus cDNAs used were of viruses, wild type (pUC18/T7 (+) HVJRz; WT), the M defect type (pHVJ-dM; dM), and the M deletion type (pHVJ-dMEA; dMEA). For the M deletion type, the results of two experiments were shown (dMEA2-1, and dMEA2-2). The wild type virus showed an activity higher than 16, whereas the other samples did not show any significant activity. The result indicates that the Sendai virus cDNA in which the M gene is deleted or is defective did not produce a virus with disseminative capability in the standard reconstitution experiment.

### Example 6. Plaque formation

[0059] CV-1 cells were plated at  $5 \times 10^5$  cells/well into a 6-well plate (Corning, microplate 6-well) and cultured overnight.

[0060] LLCMK2 cells obtained in Example 4 were diluted with PBS to  $1 \times 10^6$  cells/ml, and 100  $\mu$ l of the suspension was dispensed into 1.5 ml microtubes (Eppendorf). A portion of the dispensed suspensions was subjected to three repeats of freeze-thawing by freezing at -80°C for 10 minutes and thawing in water at room temperature for 3 minutes. Trypsin was added to all the cell suspensions at the final concentration of 0.75  $\mu$ g/ml and the mixtures were incubated at 37°C for 30 minutes.

[0061] The media were removed from the plates of CV-1 cell culture, and the cells were washed once with PBS,

then overlaid with 100  $\mu$ l of the above diluted cell suspension treated with trypsin. The mixtures were incubated for 1 hour with agitating every 15 minutes so as to spread the suspension over the whole plate. The plates were overlaid with 3 ml of 1 x MEM containing 1% agar, supplemented with 0.1% BSA, Rifampicin 100  $\mu$ g/ml, Ara C 40  $\mu$ g/ml, and trypsin 0.75  $\mu$ g/ml, that was preheated to approximately 45°C. After the agar solidified, the plates were inverted and incubated at 37°C for 5 days in 5% CO<sub>2</sub>.

[0062] Then, 1 ml of a fixative (ethanol:acetic acid = 5:1) was added onto the agar, and the plates were allowed to stand at room temperature for 90 minutes. The agar was then removed, and 200  $\mu$ l of Amido Black solution (0.5% Amido Black, ethanol:acetic acid:water = 45:10:45) was added. Immediately thereafter, the cells were washed with water and stained to count the plaque number and evaluate the shapes of plaques. The results were shown in Table 2 and Figure 7.

Table 2

Reconstitution efficiency of Sendai virus deficient or defective in the M gene			
M gene	Number of plaques		
	freeze-thawed		not frozen
	Run 1	Run 2	
wild type	38	22	14
M defect type (dM)	2	4	2
M deletion type (dMEA2-1)	27	26	60
M deletion type (dMEA2-2)	32	38	42

[0063] Virus cDNAs used are of wild type (pUC18/T7(+)-HVJRz), the M defect type (pHVJ-dM), and the M deletion type (pHVJ-dMEA). For the M deletion type, the results of two experiments (dMEA2-1 and dMEA2-2) are shown. The number of plaques was counted for both freeze-thawed and not frozen cell suspensions. The plaque number reflects the number of reconstituted viruses in  $1 \times 10^5$  transfected LLCMK2 cells. Experiments using freeze-thawed samples were performed twice (runs 1 and 2). Although it was reported that reconstitution efficiency of Sendai virus decreases in a mutant virus such as an addition type in which a foreign gene was inserted into genome (Hasan et al., J. Gen. Virol. 78: 2813-2820, 1997), the M deletion type showed a comparable reconstitution efficiency to that of wild type. The M defect type showed a little lower efficiency. Freeze-thawing of the transfected cells did not significantly affect the plaque number.

[0064] As is evident from Table 2 and Figure 7, plaques that are supposed to be derived from cDNAs of the M deletion type and the M defect type were observed. The size of the plaques was much smaller than that of wild type formed in simultaneous positive control (Figure 7; WT1 and WT2). To further examine the shape of the plaques, they were stained with anti-Sendai virus polyclonal antibody, which was produced by the known method by inoculating rabbits with purified Sendai virus that was inactivated as antigens.

[0065] The plaques described in Example 6, which were fixed and stained with Amido Black, were hybridized with anti-Sendai virus antibody, and subsequently with FITC-conjugated anti-rabbit IgG or with anti-mouse IgG antibody, then observed under fluorescence stereoscopic microscope. As a result, signal was detected in plaques of wild type, the M deletion type, and the M defect type, confirming that the plaques were of Sendai viruses (Figure 8).

[0066] It was observed that the plaques of wild type cDNA were almost circular, whereas those of the M deletion type and the M defect type cDNAs were small and not circular, having irregular shapes (Figure 8).

#### Example 7. Antibody staining of plaques

[0067] To determine whether the plaques, different from those of wild type in size and shapes, are derived from cDNA of the M deletion type or the M defect type, the presence of M protein was examined. Namely, these plaques were stained with anti-M protein monoclonal antibody and observed under fluorescence stereoscopic microscope.

[0068] Immunofluorescence staining of the plaques was performed as follows.

[0069] Plaques were stained with Amido Black. The position of the plaques was examined with the naked eye and marked with a marker pen. Each well of 6-well plates that contains plaques was washed once with 1 ml of PBS, overlaid with 200  $\mu$ l of 1:40 diluted anti-M protein monoclonal antibody, and incubated at 37°C for 45 minutes in a CO<sub>2</sub> incubator. Then, the well was washed three times with 1 ml of PBS, overlaid with 200  $\mu$ l of 1:100 diluted FITC-conjugated anti-mouse IgG antibody (Cosmo Bio), and incubated at 37°C for 45 minutes in an incubator. Finally, each well was washed three times with 1 ml of PBS, overlaid with 1 ml of PBS. The staining of the marked plaques was then examined under

fluorescence stereoscopic microscope.

[0070] Thereafter, the same procedure was repeated processed using anti-Sendai virus polyclonal antibody as the first antibody and FITC-conjugated anti-rabbit IgG antibody (ICN Biomedicals) as the second antibody. The wells were then observed under fluorescence stereoscopic microscope again.

[0071] The plaques from wild type cDNA were stained with both anti-Sendai virus antibody and anti-M protein monoclonal antibody. In contrast, plaques from cDNA of the M deletion or defect type were stained with anti-Sendai virus antibody, but not with anti-M protein monoclonal antibody. This indicates that the latter plaques, lacking complete M protein, are derived from cDNA of the M deletion and defect types.

[0072] Accordingly, plaques from Sendai virus gene having a deletion or defect in the M gene were obtained. Since the virus forming plaques does not propagate in chicken eggs, the virus is supposed to be incapable of budding, i.e. not disseminative. In addition, the virus is supposed to be capable of contact infiltration as it forms plaques.

[0073] This mode of propagation is probably due to cell fusion mediated by F and HN proteins. Namely, among proteins encoded by Sendai virus, F and HN proteins are expressed as a membrane protein on the cell surface, and M protein supports F and HN proteins as an intracellular anchor in virus budding. In case of the M deletion type and M defect type, budding does not occur because the anchor is lost. However, F and HN proteins are expressed on the cell surface, and cell fusion occurs mediated by these proteins, so that virus genome can be transferred into surrounding cells. In this way, it is possible to realize virus propagation without budding (Figure 9).

#### Industrial Applicability

[0074] The present invention enables providing an RNA virus vector capable of cell infection, autonomous RNA replication, and infiltration through contact, but incapable of dissemination. By utilizing the RNA virus vector of the present invention, it is possible to carry out more efficient gene introduction and cell transplantation in gene therapy.



SEQUENCE LISTING

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<120> RNA virus vector with contact infiltration capability

10 <130> D3-002PCT

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15 <150> JP 1998-227398

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<223> Description of Artificial Sequence: A primer used for generating a stop codon within M gene of SeV

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# Claims

1. An RNA molecule that forms a complex capable of cell infection, autonomous RNA replication, and contact infiltration, but incapable of dissemination, wherein said RNA comprises genes involved in contact infiltration and autonomous RNA replication, but no or inactivated genes involved in dissemination.
2. The RNA molecule according to claim 1, wherein genes encoding a protein that interacts with the envelope and the virus core is deleted or inactivated.
3. The RNA molecule according to claim 2, wherein said protein that interacts with the envelope and the virus core is Matrix protein (M protein).
4. The RNA molecule according to claim 1, wherein said RNA molecule is derived from a non-segmented (-)RNA virus.
5. The RNA molecule according to claim 1, wherein said RNA molecule is derived from Sendai virus and comprises no or inactivated gene encoding M protein.
6. The RNA molecule according to any one of claims 1 to 5, wherein said RNA molecule comprises a foreign gene.
7. A cell comprising the RNA according to any one of claims 1 to 6, wherein the cell is capable of allowing said RNA to replicate and transmitting said RNA to another cell through contact infiltration.
8. A DNA molecule comprising a template DNA for transcribing the RNA according to any one of claims 1 to 6 *in vitro* or in cells.

9. A complex capable of cell infection, autonomous RNA replication, and contact infiltration, but incapable of dissemination, wherein said complex comprises the RNA molecule of any one of claims 1 to 6 and a virus structure without nucleic acid.

10. A kit comprising a) the RNA according to any of claims 1 to 6, a cRNA of said RNA, or a unit that is capable of biosynthesizing said RNA or said cRNA, and b) a group of enzymes required for replication of said RNA or said cRNA, or a unit that is capable of biosynthesizing said enzymes.

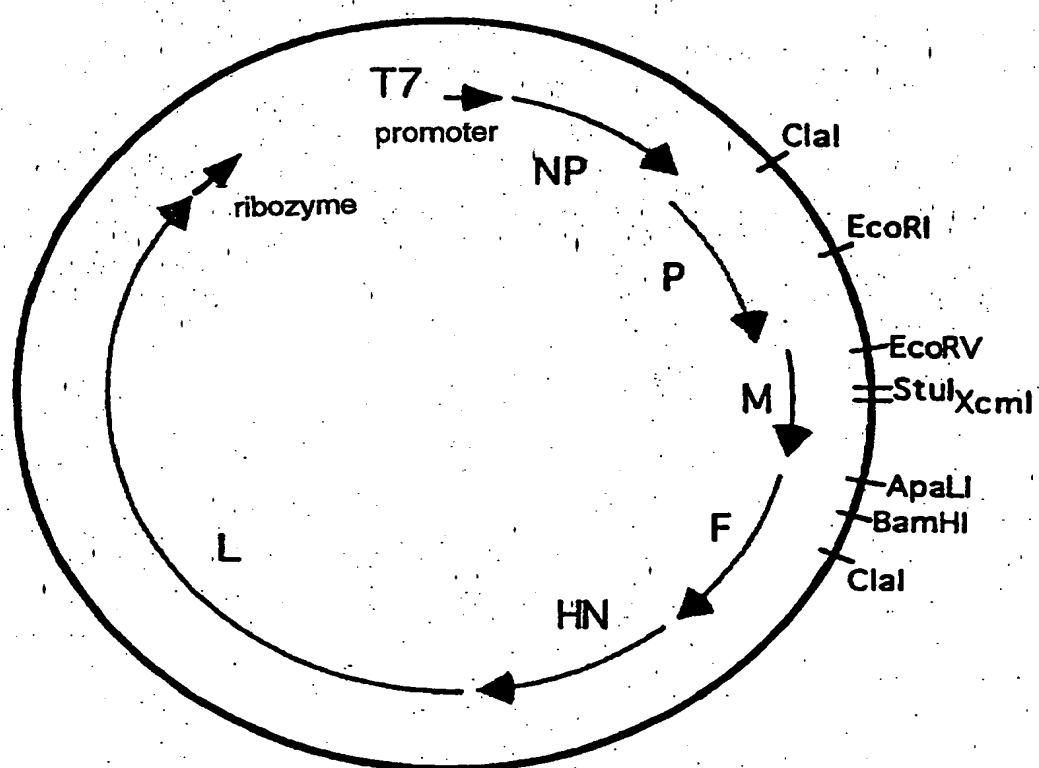
11. The kit according to claim 10, wherein a) is the RNA according to claim 5 or 6, a cRNA of said RNA, or a unit that is capable of biosynthesizing said RNA or said cRNA, and b) is all of the proteins, NP, P/C, and L of Sendai virus, or a unit that is capable of biosynthesizing said proteins.

12. A method for producing the complex according to claim 9, wherein said method comprises introducing into a host a) the RNA of any one of claims 1 to 6, a cRNA of said RNA, or a unit that is capable of biosynthesizing said RNA or said cRNA, and b) a group of enzymes required for replication of said RNA or said cRNA, or a unit that is capable of biosynthesizing said enzymes.

13. The method according to claim 12, wherein a) is the RNA of claim 5 or 6, a cRNA of said RNA, or a unit that is capable of biosynthesizing said RNA or said cRNA, and b) is all of the proteins, NP, P/C, and L of Sendai virus, or a unit that is capable of biosynthesizing said proteins.

14. A method for expressing a foreign gene, wherein said method comprises inoculating the cell of claim 7 into a nonhuman mammal and allowing a cell contacted with said cell to express a foreign gene.

Fig. 1.



PUC18/T7(+)/HVJRz  
18154 bp

Fig. 2

E	I	S	EcoRV	
TAAGAAAA	C	TTAGGGTGAA	AGAAATTTCA	CCTAACACGG CGCAATGGCA
GATTCCTAA	GTTCTCATAT	GAGGATAACG	GTA	CTGTGGA
GTCCGGATAA	GAAAGCCATC	CCCCACATCA	GGATTGTCAA	GGTAGGAGTC
ATGGAGTGAG	ATACCTAGAT	TTATTGCTCT	TGGGTTTCTT	TGAGACACCG
CCAATCTAGG	GAGCGTATCT	GACTTGACAG	AGCCGACCAG	CTACTCAATA
GGTCGTTACC	CATAGGTGTG	GCCAAATACT	ACGGGACTGA	TCAGGAACTC
GCACCGATCT	CAGAATTACG	GTGAGGAGGA	CTGTTTCGAGC	AGGAGAGATG
TGGTGGATTC	GATTGGTGCT	CCACTCCTAC	CATGTCAGG	CAGGCTGAGA
TA	TTAATGC	AAACAAGGTC	GCACTAGCTC	CCCAATGCCT
GACTCAGAGT	GGTGTGTC	AATGGGACAT	CTCTAGGGGC	AATCACCATA
CAAAGACCCT	TGCAGACCTT	GCATTGCCCA	ACTCTATATC	CGTTAATTTA
TCAAGACCGG	GATCTCCACA	GAACAAAAGG	GGTACTCCC	AGTACTTGAT
AGAAAAAGCT	CAATTTTATG	GTGCACCTCG	GGTTGATCAG	GAGAAAGGTC
ACTCTGTTGA	GTA	CTGCAAG	AGCAAGATTG	AGAGAATGCG
TAATCGGCGG	TATAAGCTTC	CATGTTTCAGG	TTATTGGGAC	ACTATCTAAG
GTCAGCTCGC	ATGGAAGAGG	GCAGTCTGCT	TCCCATTAAT	GGATGTGAAT
ACATGGTGAT	TTGGGCGGCA	TCTGTAGAAA	TCACAGGCGT	CGATGCGGTG
CCATCCCTCG	TGATTTC	CGC	TACTACCCTA	ATGTTGTGGC
GAAAGCTGTA	AA	GTGCAAC	CATCAGAGAC	CTGCGACAAT
GGCAGTCGGA	GCCACCGGT	CCTCCTTGT	CTTAA	TAAG AAAA
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				GGATAAG

Fig. 3

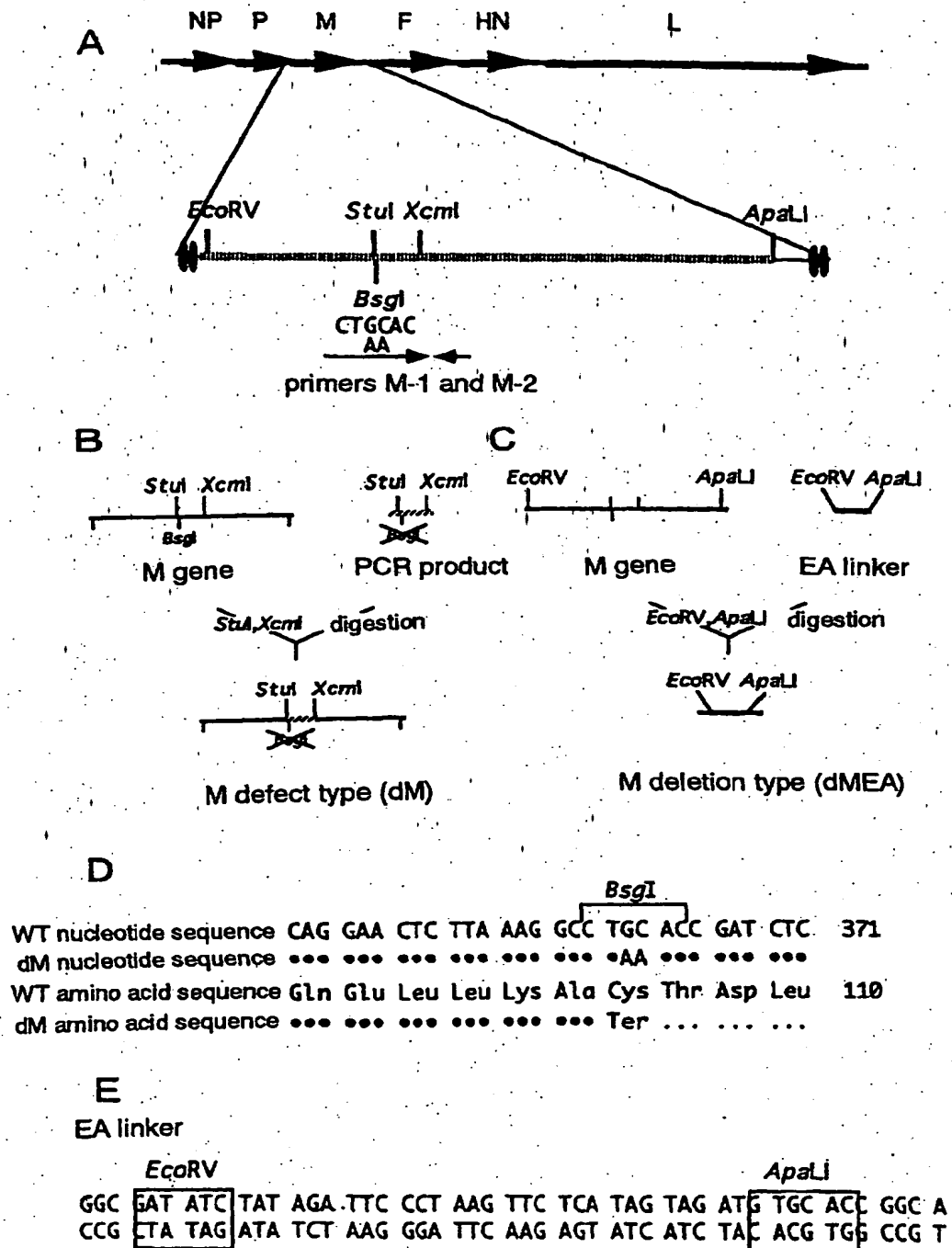


Fig. 4

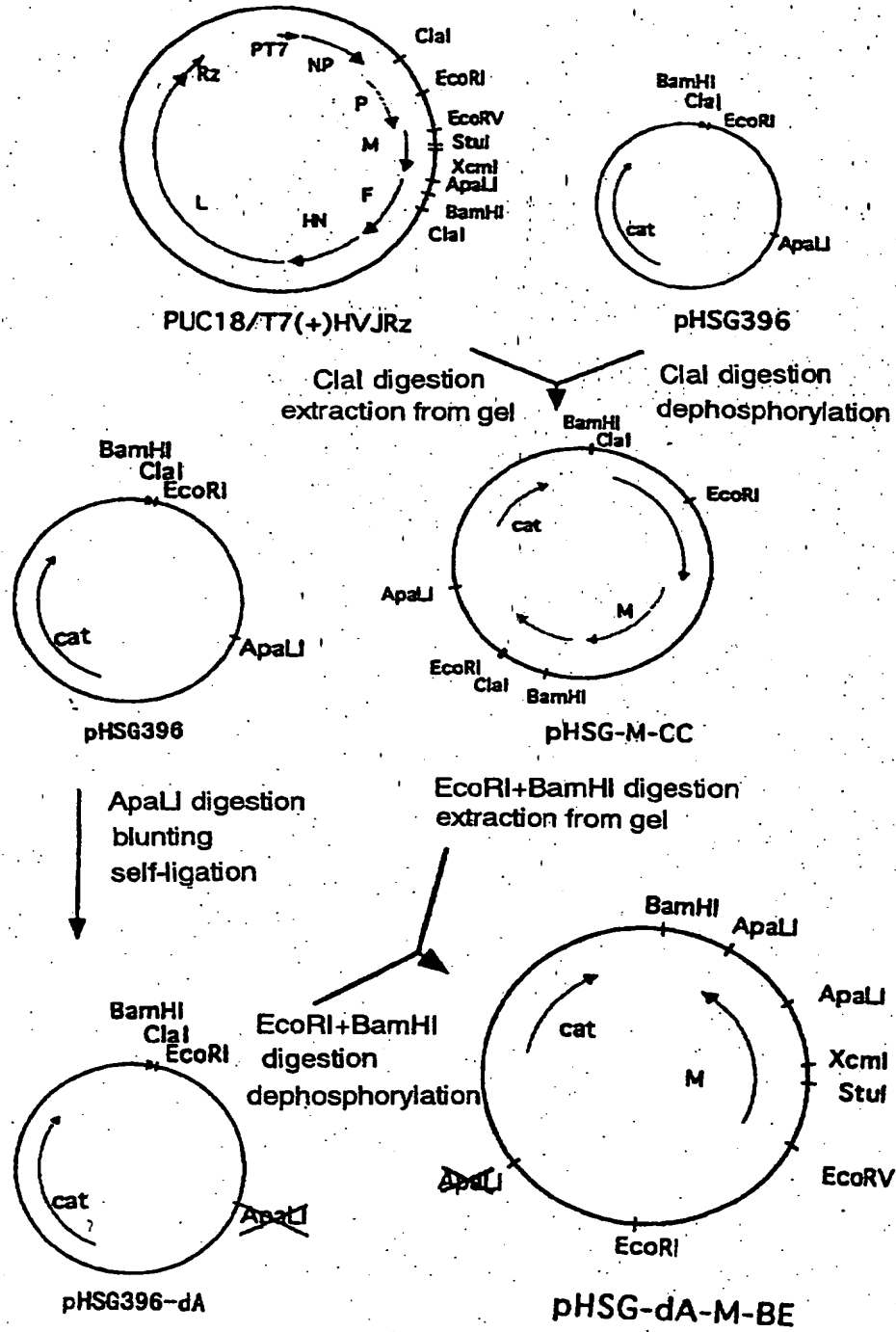


Fig. 5

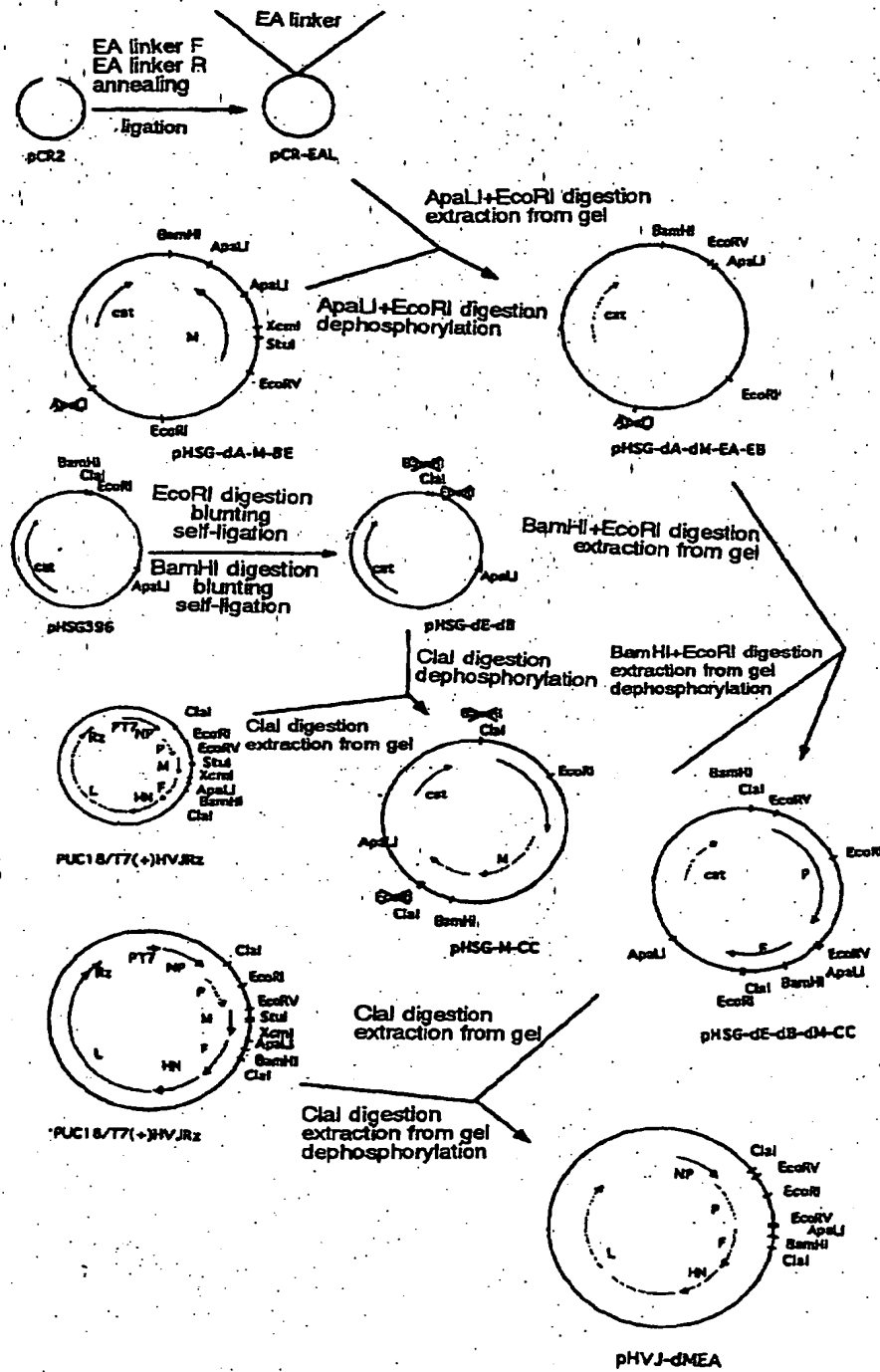




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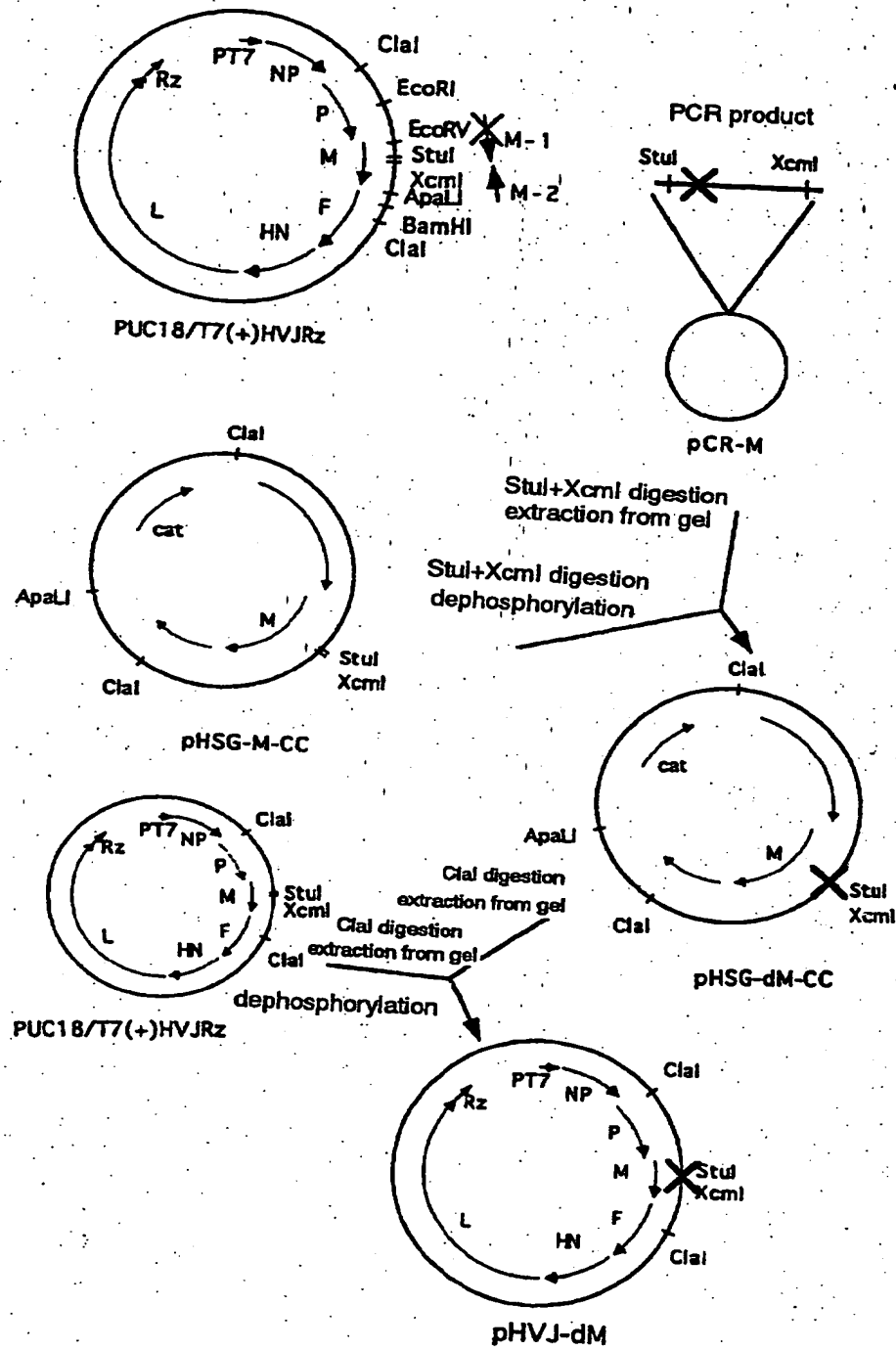
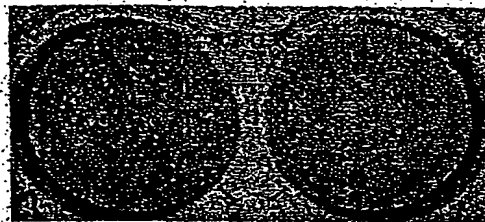


Fig. 7



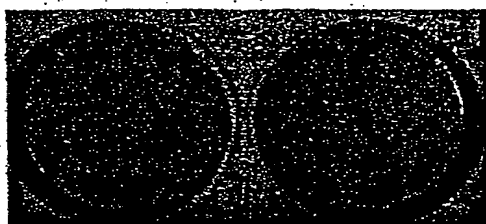
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dMEA2-1



dMEA2-2



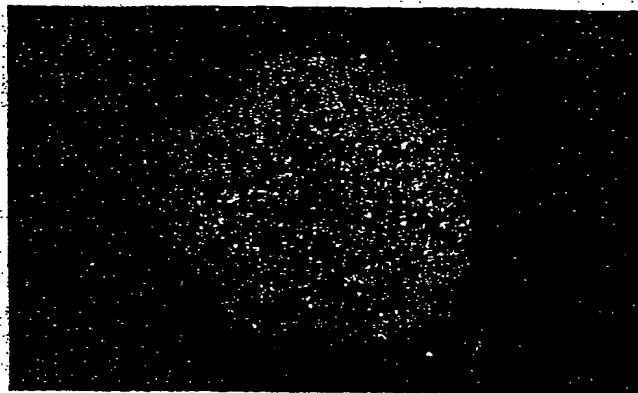
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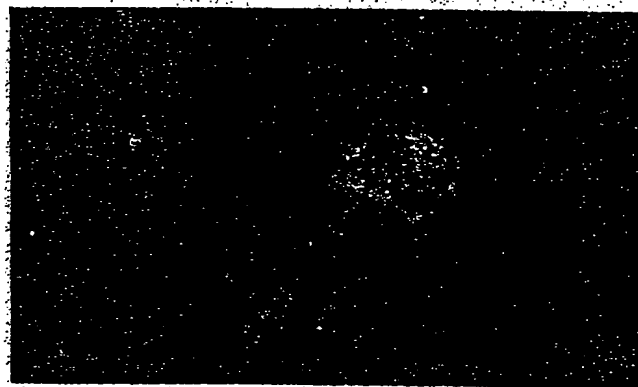
dM

Fig. 8

WT



dM



dMEA

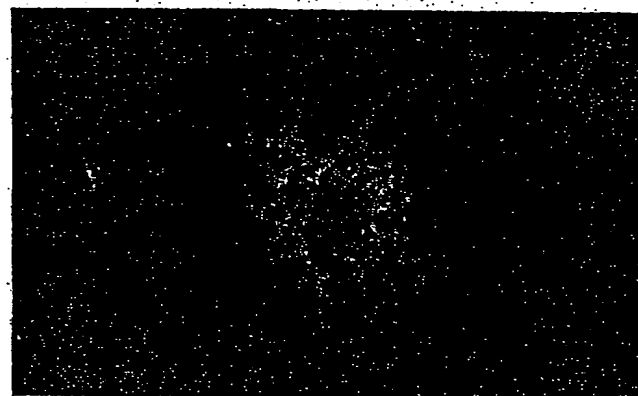
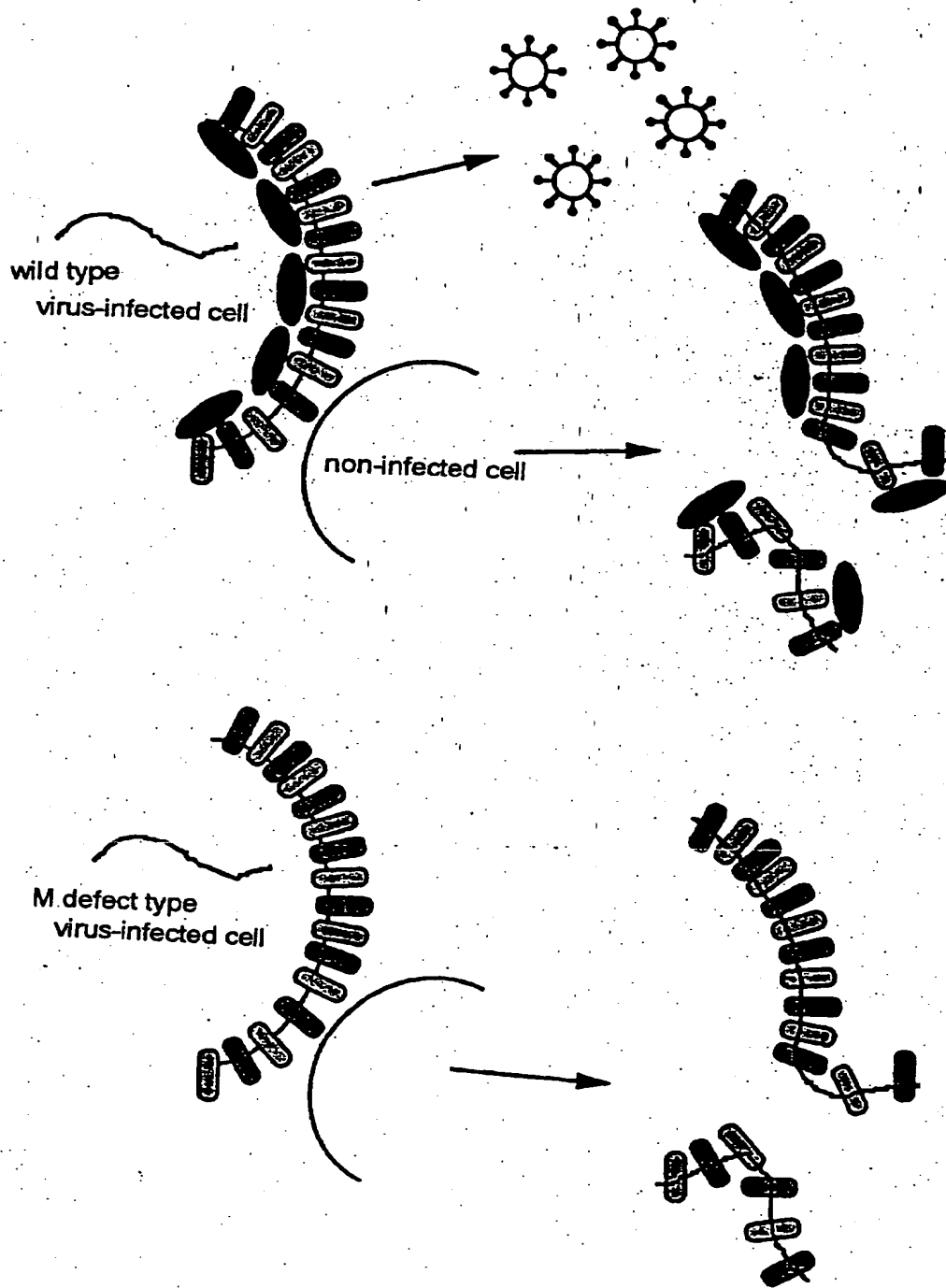


Fig. 9



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP99/04333

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int.Cl. <sup>6</sup> C12N15/40, C12N7/04, C12N5/06, A61K48/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
Int.Cl. <sup>6</sup> C12N15/40, C12N7/04, C12N5/06, A61K48/00		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI (DIALOG), BIOSIS (DIALOG)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Genevieve Mottet et al, "A Sendai Virus Vector Leading to the Efficient Expression of Mutant M Proteins Interfering with Virus Particle Budding" Virology (1996) Vol. 221 No. 1 P.159-171	1-14
X	WO, 97/16538, A1 (DNAVEC Research Inc.), 9 May, 1997 (09. 05. 97) & EP, 864645, A	1-14
A	Toru Kondou et al., "Temperature-sensitive Phenotype of a Mutant Sendai Virus Strain Is Caused by Its Insufficient Accumulation of the M Protein" The Journal Of Biological Chemistry (1993) Vol. 268 No. 29 P.21924-21930	1-14
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 6 September, 1999 (06. 09. 99)		Date of mailing of the international search report 21 September, 1999 (21. 09. 99)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

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